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Altitudinal variations in chemical profile and antioxidant activities of lentil (*Lens culinaris* Medik.) genotypes

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Abstract
The present investigation was carried out to evaluate the altitudinal variation between two lentil (*Lens culinaris* Medik.) genotypes on the basis of their chemical profile, total phenolic content and antioxidant activitiy. Pulse samples were collected from two different altitudes of Uttarakhand state. The chemical profiling was done via Gas chromatography-Mass spectrometry analysis. A total of 45 and 33 phytoconstituents accounting for 76.51% and 81.03% of the total extract has been identified in the sample from lower altitude and higher altitude respectively. Antioxidant activity was determined in terms of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging and metal chelating assays. Results revealed that the genotype from higher altitude exhibits higher antioxidant potential than the genotype from lower altitude. A significant positive correlation has been observed between altitude and total phenolic content, whereas a significant negative correlation between altitude and 50% inhibitory concentration (IC_{50}) of both genotypes.

Keywords: Altitude, antioxidant, chemical profile, lentil, phenolic content

1. Introduction
Plant-based foods have always been a central part of our traditional diet system. In recent years, these food materials have been extensively investigated for their nutritional as well as medicinal advantages. Legumes are one of the important plant-based foods and also known as the oldest cultivated crops. As a chief food staple, legumes are being utilized in most part of the world including South Asia, Europe, the Middle East and Africa [1]. In addition to an important functional and nutritive food source, they have also captured a huge deal of attention because of their enormous physiological profits, nutritional properties, functional components and significant health-promoting effects in relation to diseases prevention and cure [2]. They are well known as a rich source of micro and macronutrients, proteins, carbohydrates, dietary fibers, vitamins and minerals. In addition to these nutritional components, lentil also consists of some bioactive components such as enzyme inhibitors, oligosaccharides, phytates, lectins and phenolics which play a key role in several metabolic activities in humans [3].
Among food legumes, lentil is considered as one of the most important in view of its enormous pharmacological attributes as a diet source. Lentil is considered to be a good source of antioxidant due to the high amount of bioactive phytochemicals. A range of different phytochemicals such as alkaloids, glycosides, phytosterols, terpenoids, soluble and insoluble-bound phenolics, flavonoids, tannins, carotenoids, tocopherols, phytic acids and saponins are found in lentil which imparts the health promoting effects on its consumption [4, 5]. Antioxidants are the agents which contribute to the prevention of cells and many macromoleculs from the oxidative damages caused by free radicals. These oxidative damaged are considered to be one of the major factors involve in the generation of several chronic diseases in humans such as cardiovascular diseases, disorders like Alzheimer's and Parkinson's diseases, cancer, and diabetes [6]. Plant secondary metabolites such as phenolics are known to possess antioxidant potential which prevent or delay oxidative damages and in turn prevent the initiation of oxidative stress associated diseases in humans [7]. Prevention of several chronic diseases can be limited by the consumption of food rich in phenolics [8].
Many studies have reported the contribution of lentil in the prevention of diseases caused by free radical and hence considered as a good dietary source of antioxidant. The main objectives of this study were to check altitudinal effect on the chemical composition, phenolic content and antioxidant property of the lentils.

The findings of the study can be valuable to gain information about the effect of altitudinal variations on various chemical properties of lentils.

2. Material and Methods

2.1 Plant material

The seeds of lentil genotypes were collected from two different altitudes of Uttarakhand. Sample from lower altitude were obtained from Crop Research Center, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand (344 m above sea level) whereas sample from high altitude was collected from Pithoragarh district of Uttarakhand (1514m above sea level).

2.2 Preparation of extract

Seed samples were ground (10 g each) and homogenized with methanol (1:10, w/v) and kept for 72 hours for complete extraction with occasional stirring. Samples were then centrifuged at 4000 rpm and supernatant was pooled followed by complete evaporation of solvent. The extracts obtained, were stored at -20 °C for further analysis.

2.3 Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out at Advanced Instrumental Research facility, Jawaharlal Nehru University (JNU), New Delhi using gas chromatograph HP 6890 with mass selective detector MS 5973 (Agilent technologies, USA).

2.4 Identification of compounds

The compounds were identified by comparing their mass spectrum with those given in NIST-MS, FFNSC Wiley Library. The KI values of compounds were compared with earlier literature reports [9].

2.5 Estimation of total phenolic content

Total phenolic content was estimated by using Folin–Ciocalteu reagent (FCR) with slight modification [10]. 0.5 ml of appropriately diluted extract was mixed with 0.5 ml of Folin–Ciocalteu Reagent (FCR) followed by the addition of 1 ml of saturated sodium carbonate solution to neutralize the reaction and final volume of mixture was made up to 10 ml with distilled water. The reaction mixtures were incubated in dark for 35 minutes at 24 ℃. The mixtures were then centrifuged at 4000 rpm for 10 min and absorbance of supernatant was read at 725 nm against reagent blank. Total phenolic content was calculated by establishing standard curve using different concentrations of gallic acid and results were recorded as mg gallic acid equivalents (mg $GAEg^{-1}$).

2.6 Estimation of DPPH radical scavenging activity

DPPH radical scavenging activity was estimated by using standard protocol [11]. 1 ml of different concentrations of extracts (200-1000 µg/ml) was mixed with 5 ml of 0.4 mM DPPH solution followed by incubation in dark at room temperature for 30 minutes. The absorbance of mixtures was recorded at 517 nm against blank. The control was subjected to the same procedure. Percent DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ inhibition} = [(A - B) / A] \times 100$$

Where, A and B are the absorbance values of control and sample, respectively. Gallic acid, BHT (butylated hydroxyl toluene) and ascorbic acid were used as standard compounds. The scavenging activity was expressed as 50% inhibitory concentration (IC_{50}) which was calculated by plotting straight line between concentration and percent inhibition.

2.7 Estimation of hydoxyl radical scavenging activity

Hydroxyl radical scavenging activity was estimated by using standard method [12]. 1 ml of different concentrations of extracts (200-1000 µg/ml) was added to reaction mixture containing 60 µl of 0.1 mM $FeSO_4.7H_2O$, 90 µl of 1 mM 1,10-Phenanthroline and 2.4 ml of 0.2 M phosphate buffer (pH 7.8). Reaction was initiated by adding 150 µL of 0.17 M hydrogen peroxide to the reaction mixture. The absorbance of mixture was measured at 536 nm after 5 minutes of incubation at room temperature. The control was subjected to the same procedure. Ascorbic acid was taken as standard. Percent hydroxyl radical scavenging activity was calculated by the following equation:

$$\% \text{ inhibition} = [(A - B) / A] \times 100$$

Where, A and B are the absorbance values of control and sample, respectively. Ascorbic acid was used as standard compound and the scavenging activity was expressed by calculating IC_{50} value.

2.8 Estimation of metal chelating activity

Metal chelating activity of extracts was determined by the standard method [13]. The different concentrations of extract (200-1000 µg/ml) were mixed with 0.1 ml of 2 mM $FeCl_2.4H_2O$, and 0.2 ml of 5 mM ferrozine. The final volume of mixture made up to 5 ml by methanol and allowed to react for 10 minutes. Absorbance of samples and control was measured at 562 nm. Percent chelating activity was calculated by the following equation:

$$\% \text{ inhibition} = [(A - B) / A] \times 100$$

Where, A and B are the absorbance values of control and sample respectively. EDTA was used as standard compound and the scavenging activity was expressed as IC_{50} value calculated by plotting a straight line between concentration and percent chelating activity.

2.9 Statistical analysis

Correlation between altitude, total phenolic content, DPPH radical scavenging activity, hydroxyl radical scavenging activity and metal chelating activity was determined by SPSS (Statistical Package for Social Science) software.

3. Result and discussion

3.1 Chemical profiling

The yield of methanolic extracts collected from lower and higher altitude of Uttarakhand was 2.43 % and 2.42 %, respectively. The comparative chemical composition of lentil genotypes is presented in Table 1.

Table 1: Comparative chemical composition evaluation of lentil samples from lower and higher altitude regions

S. No.	Compounds	Lower altitude (%)	Higher altitude (%)
1	Limonene	0.34	-
2	4-methoxyphenol	0.11	-
3	3,7-dimethyl-6-octenyl formate	-	0.11
4	1-terpinen-4-ol	0.05	-
5	Terpineol	0.12	0.13
6	β-citronellol	0.06	-
7	Dimethyl hexanedioate	-	0.13
8	Geranial	0.11	0.33
9	1,3,4-eugenol	0.14	0.20
10	Phenoxybenzene	-	0.03
11	Verdyl acetate	0.06	0.07
12	Caryophyllene	0.04	-
13	(E)-β-Famesene	0.08	0.16
14	Lily aldehyde	-	0.03
15	α-curcumene	0.06	-
16	Elemicin	-	0.02
17	Methyl laurate	0.08	0.08
18	Elemol	0.05	0.07
19	3-Methyldecanoic acid	-	0.20
20	3-methylquinoline	0.21	-
21	*α-santalol*	0.17	-
22	Methyl myristate	0.16	-
23	β-santalol	0.09	0.17
24	Mome inositol	0.48	-
25	Palmitic acid	0.11	0.15
26	Glycol monosalicylate	-	0.04
27	Methyl pentadecanoate	0.06	-
28	Neophytadiene	0.04	-
29	Diisobutyl phthalate	0.20	-
30	Nonadecane	0.18	-
31	Methyl acetyl ricinoleate	8.22	9.99
32	Heptadecanoic acid	4.54	3.45
33	Methyl heptadecanoate	0.10	0.11
34	Ethyl heptadecanoate	-	0.03
35	Methyl linoleate	13.93	-
36	Methyl 11-octadecenoate	20.37	25.06
37	Methyl 16-methylheptadecanoate	-	0.09
38	Methyl stearate	2.85	2.22
39	Methyl oleate	-	3.52
40	Linoelaidic acid	-	0.09
41	cis-Vaccenic acid	16.83	7.53
42	Stearic acid	0.91	-
43	Methyl (11E,14E)-11,14-octadecadienoate	1.62	18.26
44	Linoelaidic acid	0.43	-
45	N-(2-Hydroxyethyl)decanamide	0.04	-
46	Methyl heneicosanoate	0.92	-
47	Methyl 12-hydroxy-9-octadecenoate	-	5.90
48	Methyl tricosanoate	0.10	-
49	Methyl icosanoate	-	0.18
50	Methyl tetracosanoate	0.56	1.68
51	Squalene	0.07	-
52	2-methyloctacosane	0.04	-
53	Methyl triacontanoate	0.16	-
54	Caprylic acid monoethanol amide	-	0.04
55	γ-tocopherol	0.47	-
56	(6Z,9Z)-6,9-Pentadecadien-1-ol	-	0.07
57	Methyl docosanoate	-	0.89
58	Stigmasterol	0.19	-
59	Methyl commate E	0.10	-
	Total	75.45	81.03

A total of 43 and 33 components representing about 75.45 % and 81.03 % of the total extract have been identified from lower and higher altitude respectively. The major constituents identified from low altitude region were methyl 11-octadecenoate (20.49%), cis-vaccenic acid (15.58%), methyl (11E,14E)-11,14-octadecadienoate (13.28%), methyl palmitate (8.18%), 2, 3-Dihydroxypropyl elaidate (5.36%) and palmitic acid (3.28%), whereas from high altitude region were methyl 11-octadecenoate (25.06%), methyl (11E,14E)-11,14-octadecadienoate (18.26%), methyl acetyl ricinoleate (9.99%), cis-vaccenic acid (7.53%), methyl 12-hydroxy-9-octadecenoate (5.90%), methyl oleate (3.52%) and heptadecanoic acid (3.45%) as major constituents.

Results revealed that both lentils have different quantitative and qualitative makeup of major and minor constituents. Fatty acid and fatty esters were found as the dominating components in both samples. In both the lentil sample, methyl 11-octadecenoate was identified as the major component. Methyl acetyl ricinoleate, heptadecanoic acid and methyl stearate were present in similar amount in both samples. A major component methyl linoleate was identified only in the sample from low altitude region while methyl 12-hydroxy-9-octadecenoate was identified from high altitude region. The content of cis-vaccenic acid was found higher in lower altitude, whereas high amount of methyl (11E, 14E)-11,14-octadecadienoate was observed from high altitude. In addition to major constituents, some minor and trace components were also identified in both lentil samples. A large variation in the chemical composition of both samples has been found. Paucean *et al.* [4] reported lentils as a rich source of fatty acids, esters and several volatile classes of compounds like alcohol, aldehyde, ketone, alkanes and some nitrogen compounds.

3.2 Total phenolic content

Total phenolic content was observed as 6.81±0.15 mg $GAEg^{-1}$ and 12.14±0.67 mg $GAEg^{-1}$ from low altitude and high altitude, respectively. Earlier studied of Oomah *et al.* [14] and Han and Baik [15] support the present results.

3.3 DPPH radical scavenging activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method has been extensively used as a quick and reliable assay to assess *in-vitro* antioxidant activity of plant extract. DPPH is a stable radical which gets reduced in the presence of an antioxidant by accepting an electron or hydrogen and become a stable diamagnetic molecule with the visible change in colour from purple to yellow. The DPPH radical scavenging activity of lentil extracts was represented in Fig 1. The IC_{50} value of sample from low altitude was observed as 892.35±2.59 µg/ml and from high altitude 686.68±3.55 µg/ml. Lower IC_{50} denotes the higher antioxidant potential. Sample from high altitude shows a good DPPH radical scavenging activity in comparison to sample from low altitude.

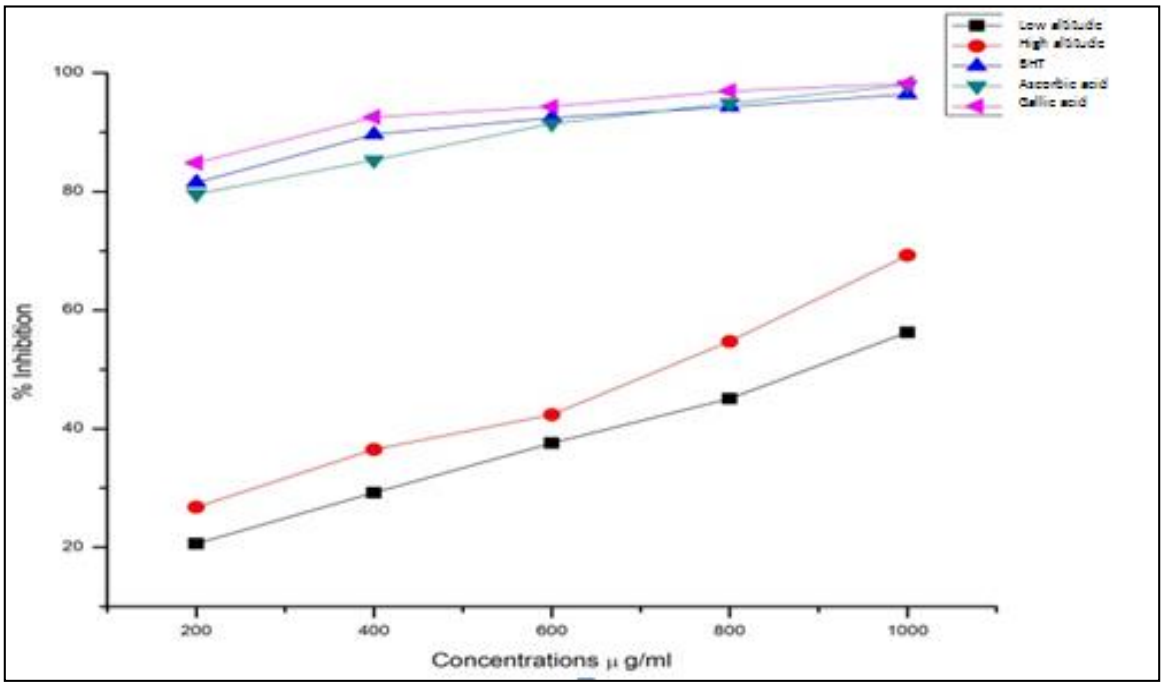

Fig 1: Altitudinal variation in DPPH radical scavenging activity

3.4 Hydroxyl radical scavenging activity

Hydroxyl radical is one of the most reactive and short lived oxygen species (ROS) generates due to the reaction between superoxide anion and hydrogen peroxide in the presence of iron catalyst via Fenton reaction [16]. The hydroxyl radical scavenging activity was represented in Fig 2. The IC_{50} value of sample from low altitude was observed as 960.8±2.65 µg/ml and from high altitude 612.27±5.79 µg/ml. High hydroxyl radical scavenging potential was observed in sample from high altitude region.

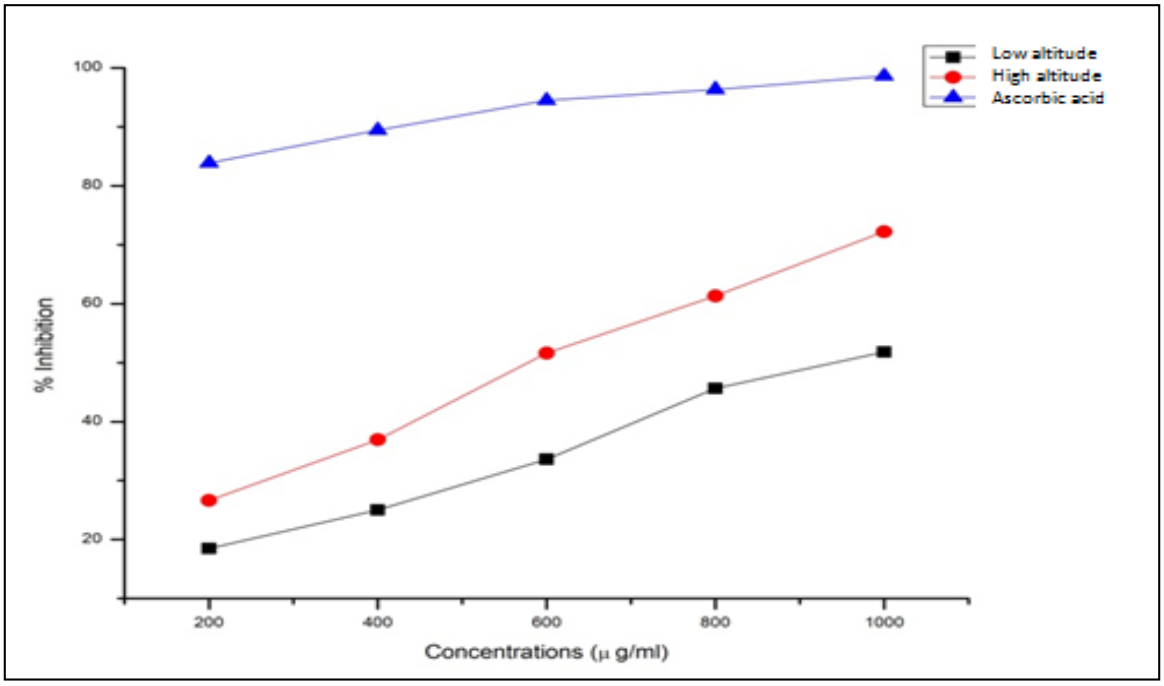

Fig 2: Altitudinal variation in hydroxyl radical scavenging activity

3.5 Metal chelating activity

The metal chelating potential of plant extracts depend on their ability to form complexes with the transition ions via chelation. The role of chelating agent is to stabilize the metal ions and in turn inhibit the formation of free radical. A red coloured ferrozine complex $Na_4Fe(ferrozine)_3$ was formed by the binding of ferrozine with ferrous ion. In the presence of an antioxidant, the chelation power reduces and causes a decrease in the intensity of red coloured ferrozine complex as antioxidant competes with ferrozine to form complex with ferrous ion [17]. Result revealed that the extract from higher altitude region has more ability to chelate ferrous ion and hence act as a good antioxidant agent. The IC_{50} of hydroxyl radical found was found as 954.01±3.43µg/ml from low altitude and 664.84±4.48 µg/ml from higher altitude.

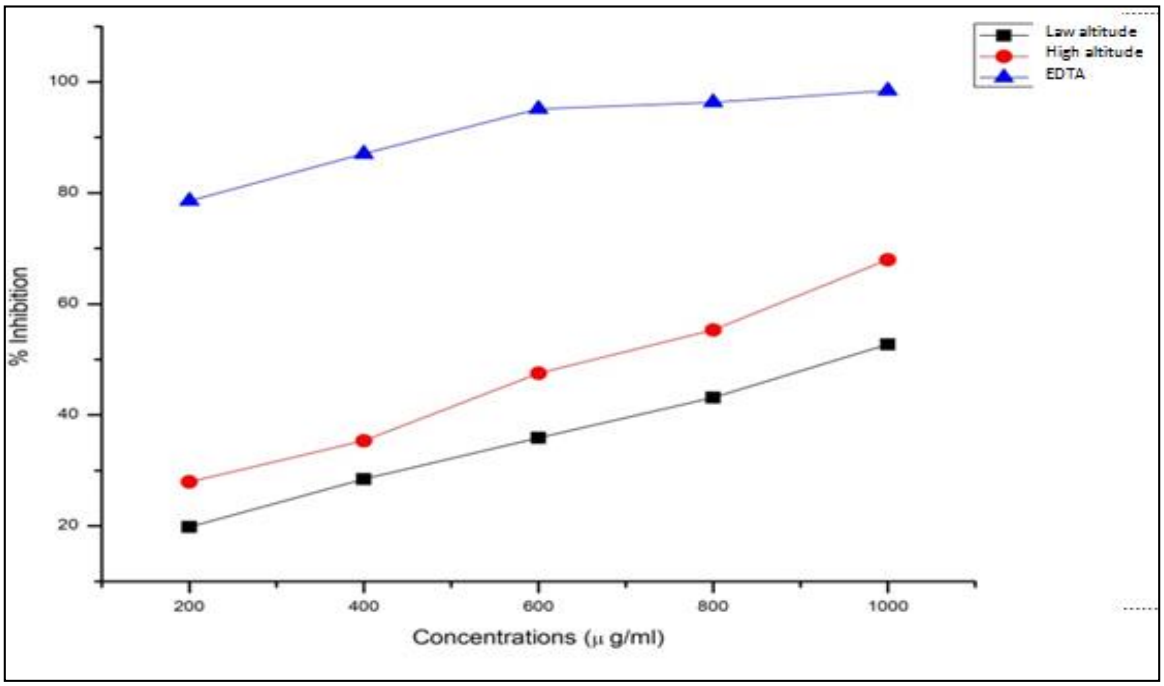

Fig 2: Altitudinal variation in metal chelating activity

3.6 Correlation study

The correlation between various parameters was presented in Table 2. Result showed a significant positive correlation between altitude and total phenolic content whereas a strong negative correlation between altitude and IC_{50} values of DPPH radical scavenging assay, hydroxyl radical scavenging assay and metal chelating assay (α=0.01).

Lentil sample from the higher altitude region consist of a high total phenolic content as well as high antioxidant potential. Results revealed that lentil sample with higher total phenolic content impart a strong antioxidant activity. Based on the highly significant correlation total phenolic content can be used as a best predictor of antioxidant activity. In addition to phenolic components, a synergistic effect of various components detected through GC-MS analysis contributes to total antioxidant activity of both lentil samples. Lu and Foo [18] suggested that the antioxidant activity of natural sources is due to the synergistic effect of their antioxidant components which together contributes to the health promoting effect in terms of radical scavenging and prevention from oxidative damages.

Table 2: Correlation between various parameters studied

	Altitude	Total phenolic content	IC_{50} DPPH	IC_{50} Hydroxy radical scavenging activity	IC_{50} Metal chelating activity
Altitude	1	1.000**	-1.000**	-1.000**	-1.000**

4. Conclusion

From the present investigation it could be concluded that altitudinal variation affects the chemical composition as well as total phenolic content of the lentils which in turn directly affect the antioxidant potential. In addition to several health benefits, lentils can be used as an antioxidant agent. Finding of the present study could form a base for the evaluation of other pharmacological benefits from sample of higher altitude region.

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